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PAIN

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# Effects of the bradykinin $B_1$ receptor antagonist des-Arg$^9$[Leu$^8$]bradykinin and genetic disruption of the $B_2$ receptor on nociception in rats and mice

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**Abstract**

The contributions of $B_1$ and $B_2$ bradykinin receptors to acute and chronic inflammatory hyperalgesia were examined using the peptide $B_1$ receptor antagonist des-Arg$^9$[Leu$^8$]bradykinin and transgenic *Bk2r*$^{-/-}$ mice. In normal rats and mice, des-Arg$^9$[Leu$^8$]bradykinin (30 nmol/kg i.v. or s.c.) inhibited carrageenan-induced hyperalgesia and the late phase nociceptive response to formalin. The active dose range was narrow, suggesting partial agonist activity of this peptide. In rats with monoarthritis, des-Arg$^9$[Leu$^8$]bradykinin (up to 30 nmol/kg i.v.) failed to reduce the number of vocalisations elicited by gentle flexion and extension of the inflamed limb; however, hyperalgesia was exacerbated by administration of the $B_1$ receptor agonist des-[Arg$^9$]bradykinin (100 nmol/kg i.v.), consistent with other evidence for local induction of $B_1$ receptors during adjuvant-induced arthritis. The nociceptive response to intraplantar injection of bradykinin (10 nmol) and hyperalgesia induced by carrageenan (0.6 mg) were absent in *Bk2r*$^{-/-}$ mice, indicating that stimulation of $B_2$ receptors is an essential step in the initiation of some nociceptive and inflammatory reactions. However, the nociceptive response to formalin (2.5% intraplantar), including inhibition of the late phase by des-Arg$^9$[Leu$^8$]bradykinin (0.3 nmol), and induction of thermal hyperalgesia by Freund's adjuvant (0.1%) appeared intact in *Bk2r*$^{-/-}$ mice. These findings support other evidence for an involvement of $B_1$ receptors in inflammatory hyperalgesia and suggest that $B_1$ receptor antagonists may be clinically useful as anti-inflammatory and analgesic drugs. 

*Keywords:* Bradykinin $B_1$ and $B_2$ receptors; Transgenic mouse; Formalin paw; Carrageenan; Freund's adjuvant

## 1. Introduction

Bradykinin is believed to be a primary mediator of pain and inflammation, acting both as a potent and direct activator of nociceptors, and as an inducer of prolonged inflammatory hyperalgesia. Bradykinin is released in damaged tissues from blood-borne kininogen precursors by the action of kallikreins, and is rapidly inactivated by proteolytic enzymes yielding des-[Arg$^9$]bradykinin as a major active metabolite. In addition to its algogenic and vasodilator properties (Coffman, 1966; Whalley et al., 1987), bradykinin triggers an inflammatory positive feedback cycle, stimulating the release of prostaglandins and cytokines which in turn amplify the responsiveness of the inflamed tissue to bradykinin (Regoli and Barabe, 1980; Burch et al., 1989). Elevated levels of circulating bradykinin have been demonstrated in patients with rheumatoid arthritis, confirming the involvement of bradykinin in pathophysiological processes of inflammatory disease (Hargreaves et al., 1988). Bradykinin is also believed to be a mediator of the bone resorption seen in chronic inflammatory conditions such as periodontitis, osteomyelitis and rheumatoid arthritis (Ljunggren and Lerner, 1990). These observations provide a compelling rationale for the development of bradykinin antagonists as powerful analgesic and anti-inflammatory drugs.

The actions of bradykinin are mediated via two distinct receptors designated $B_1$ and $B_2$. Both receptors have been cloned (Hess et al., 1992; Menke et al., 1994). Bradykinin itself is thought to act mainly through $B_2$ receptors that are constitutively expressed in a wide range of tissues, includ-

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ing nociceptive neurones, sympathetic nerves, vascular smooth muscle and immune cells (macrophages and neutrophils). The $B_1$ receptor shows 10–50 fold higher affinity for the metabolite des-[Arg$^9$]bradykinin than for bradykinin and is expressed in low abundance in normal tissues; the de novo synthesis of $B_1$ receptors is increased over time following tissue damage and exposure to inflammatory agents (Regoli and Barabe, 1980; Regoli et al., 1981). The inducible nature of $B_1$ receptors at the site of injury makes this an attractive target for drug development since antagonists of this receptor might be expected to cause minimal disruption of normal physiology in non-inflamed tissues and exhibit few unwanted side-effects.

Preclinical studies support the proposal that $B_1$ and $B_2$ receptor antagonists may have clinical utility for the treatment of inflammation and pain. Of particular interest is the ability of the peptide $B_1$ receptor antagonist des-Arg$^9$[Leu$^8$]-bradykinin to reverse or prevent the persistent (≥24 h) mechanical and thermal hyperalgesia caused by intra-articular injection of Freund's adjuvant, or by ultraviolet (u.v.) irradiation of the skin in rats; in contrast the peptide $B_2$ receptor antagonist HOE 140 was ineffective or only weakly active in these assays (Perkins and Kelly, 1993; Perkins et al., 1993; Davis and Perkins, 1994a). Moreover, local injection of the $B_1$ agonist des-[Arg$^9$]bradykinin exacerbated hyperalgesia caused by intra-articular injection of Freund's adjuvant without affecting weight bearing by normal joints, indicating local induction of $B_1$ receptors at the site of inflammation (Davis and Perkins, 1994a). Studies of intra-articular plasma extravasation in antigen-induced arthritis also reveal an evolving role for $B_1$ receptors in the maintenance of chronic inflammation (Cruwys et al., 1994). In other studies examining the effects of u.v.-irradiation of the paws, thermal hyperalgesia in rats was further increased by intravenous injection of des-[Arg$^9$]bradykinin, and to a lesser extent by bradykinin; this effect of both agonists was reversed by des-Arg$^9$[Leu$^8$]bradykinin, but not by HOE 140, implicating $B_1$ rather than $B_2$ receptors in persistent hyperalgesia (Perkins and Kelly, 1993). These findings have led to the proposal that stimulation of the $B_2$ receptor initiates the acute nociceptive and inflammatory response to bradykinin, and that the inducible $B_1$ receptor may play an important role in the development and maintenance of chronic inflammatory hyperalgesia (Dray and Perkins, 1993).

Consistent with this proposal is the activity of peptide $B_2$ receptor antagonists such as HOE 140 and NPC 567 in acute antinociception assays involving inhibition of acetic acid-induced abdominal constriction (Steranka et al., 1987; Heapy et al., 1993), carrageenan induced thermal hyperalgesia (Costello and Hargreaves, 1989) and urate induced mechanical hyperalgesia (Steranka et al., 1987). However, other evidence suggests that $B_1$ receptors may also contribute to acute nociceptive and inflammatory responses, since des-Arg$^9$[Leu$^8$]bradykinin attenuated the late phase nociceptive response to formalin (Shibata et al., 1989; Correa and Calixto, 1993) and blocked capsaicin-induced dermal inflammation (Mantione and Rodriguez, 1990); these assays were performed ≤30 min after injection of the inflammatory agent. Moreover, the ability of carboxypeptidases to convert peptide $B_2$ receptor antag nists into $B_1$ receptor blockers (Regoli et al., 1986) suggests that the contribution of $B_1$ receptors to acute nociception and inflammation may have been underestimated.

The recent production of transgenic mice with targeted disruption of the gene encoding the $B_2$ bradykinin receptor (Borkowski et al., 1995) provides a unique opportunity to circumvent the limitations of currently available peptide bradykinin antagonists and further explore the relative contributions of the $B_1$ and $B_2$ receptor to acute and chronic noxious and inflammatory responses. Unlike wild type and heterozygous animals, mice that are homozygous for deletion of the $B_2$ receptor gene (*Bk2r*$^{-/-}$) fail to exhibit detectable binding of [$^3$H]bradykinin to membranes from the ileum or uterus, or functional responses to bradykinin in uterine and neuronal (superior cervical ganglia) tissues (Borkowski et al., 1995). The present studies characterise the nociceptive responses of *Bk2r*$^{-/-}$ mice and examine whether des-Arg$^9$[Leu$^8$]bradykinin exhibits antinociceptive activity in mutant mice lacking the $B_2$ receptor. Since many conventional nociception assays (particularly those examining mechanical hyperalgesia) cannot be directly applied to mice, the antinociceptive effects of des-Arg$^9$[Leu$^8$]bradykinin were also examined in rats in order to further define the breadth of activity of this agent.

## 2. Methods

All experiments conformed to ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983). The number of animals and intensity of noxious stimuli were the minimum necessary to demonstrate consistent effects of drug treatments or genetic manipulation. Animals were used once only and were humanely killed immediately on completion of testing.

### *2.1. Antinociceptive activity of des-Arg$^9$[Leu$^8$]bradykinin in mice and rats*

#### *2.1.1. Nociceptive behaviour elicited by intraplantar injection of formalin in mice*

Male BKTO mice (20–30 g) were habituated to individual perspex observation boxes (25 × 20 × 20 cm) for at least 2 h prior to administration of formalin. Mice received indomethacin (30 mg/kg i.p.), des-Arg$^9$[Leu$^8$]bradykinin (3, 10, 30 or 100 nmol/kg s.c.) or vehicle (saline s.c.) followed 10 min later by an intraplantar injection of formalin (20 μl of 2.5% solution in phosphate buffered saline) into one hind paw. Animals were immediately returned to their observation boxes and the duration of licking directed at the injected paw was recorded during the 20–30-min period after injec-

tion of formalin since antinociceptive effects of both indomethacin (Hunskaar et al., 1986) and des-Arg$^9$[Leu$^8$]bradykinin (Shibata et al., 1989; Correa and Calixto, 1993) have been most consistently detected during th late phase inflammatory response to formalin. Twelve animals received each dose of test compound or vehicle.

#### 2.1.2. Carrageenan-induced mechanical hyperalgesia in rats

The ability of des-Arg$^9$[Leu$^8$]bradykinin and indomethacin to reverse carrageenan-induced hyperalgesia was determined using the method described by Boyce et al. (1994). The paw pressure threshold of male Sprague-Dawley rats (90–110 g) was assessed by eliciting a vocalisation response to compression of the hind paw in a modified Ugo Basile algesiometer. Baseline nociceptive thresholds were first determined in order to permit allocation of rats into balanced groups receiving each drug treatment or vehicle. Rats then received an intraplantar injection of carrageenan (4.5 mg in 0.15 ml), or saline (0.15 ml), into one hind paw, followed 2 h later by indomethacin (10 mg/kg p.o.), des-Arg$^9$[Leu$^8$]bradykinin (1, 10, 30 or 100 nmol/kg i.v.) or vehicle (saline i.v.). The threshold paw pressure required to elicit the vocalisation response on compression of the inflamed paw was reassessed 1 h later. Mechanical hyperalgesia was defined as the difference in vocalisation threshold between rats which had received intraplantar injection of carrageenan or saline. Paw pressure scores in drug-treated rats are expressed as a percentage inhibition of the hyperalgesia induced by carrageenan. Ten animals received each dose of test compound or vehicle.

#### 2.1.3. Ankle flexion test in monoarthritic rats

Arthritis restricted to one ankle joint was induced by intra-articular injection of Freund's adjuvant as described by Butler et al. (1992). Male Sprague Dawley rats (240–270 g) received an intra-articular injection of complete Freund's adjuvant (50 μl containing 0.1% of killed *Mycobacterium butyricum*) into one ankle joint under isoflurane anaesthesia. Hyperalgesia was assessed 7 or 8 days later by the total number of vocalisations emitted following gentle flexion and extension (five manipulations in each direction) and animals with scores greater than 5 were then allocated to treatment groups so that their mean pretreatment vocalisation scores were balanced. The ankle flexion/extension test was repeated 1 h after administration of indomethacin (10 mg/kg p.o.), des-Arg$^9$[Leu$^8$]bradykinin (1, 10 or 30 nmol/kg i.v.) or vehicle (saline i.v.). Between 10 and 20 rats received each dose of test compound or vehicle.

In separate experiments, the ability of the peptide $B_1$ receptor agonist des-[Arg$^9$]bradykinin to exacerbate hyperalgesia in monoarthritic rats was examined. Rats with baseline vocalisation scores of less than 5 were employed for these studies. The flexion/extension test was performed before and 1 h after intravenous injection f des-[Arg$^9$]bradykinin (100 nm l/kg) or saline.

### 2.2. Sensorimotor function and nociception in Bk2r$^{-/-}$ mice

#### 2.2.1. Generation of Bk2r$^{-/-}$ mice

Transgenic *Bk2r*$^{-/-}$ mice of both sexes were generated by transfection of embryonic stem cells from J129 mice with a targeting vector designed to disrupt the $B_2$ receptor gene, as described previously (Borkowski et al., 1995). For experiments examining sensorimotor function and the nociceptive response to intraplantar injection of the peptide $B_1$ and $B_2$ receptor agonists des-[Arg$^{10}$]kallidin and bradykinin, *Bk2r*$^{-/-}$ mice were hybrids of the J129 and C57 strains. Control animals for these studies were heterozygous (*Bk2r*$^{+/-}$) r wild-type (*Bk2r*$^{+/+}$) J129 × C57 hybrids. In subsequent studies, *Bk2r*$^{-/-}$ mice were bred from a pure J129 line in order to provide a more homogeneous genetic background; wild-type J129 mice served as controls for these animals. Control mice were matched as closely as possible for weight, age and sex. Experimental methodologies and appropriate doses of algogens and inflammatory agents were determined in preliminary calibration experiments using normal C57 black mice.

#### 2.2.2. Spinal reflexes and tests of sensorimotor function

At the time of testing, transgenic animals were 24–27 weeks of age. *Bk2r*$^{-/-}$ mice ($n$ = 12) and wild-type or heterozygous ($n$ = 10) mice were evaluated in a series of neurological tests examining the integrity of spinal reflexes and sensorimotor skills. Spinal reflex tests included placement of animals in a supine position and observing the righting reflex; allowing mice to grasp a wire grid with their forepaws in order to assess the hindlimb step reflex; suspending mice by the tail and lowering them towards a bench surface to observe the forelimb placing reflex; and examining reflex hindlimb extension when lifting animals by the tail. Sensorimotor co-ordination was assessed by recording the time (maximum cut-off 120 s) for which mice were able to remain on square and round balancing beams (10-mm wide and 6-mm diameter, respectively) or on a rota-rod treadmill revolving at 18 r.p.m. (Ugo Basile). Finally, swimming ability was assessed by placing the animals at one end of a tank (1 × 0.15 × 0.1 m) containing warm water (22°C) and recording the time taken to escape onto a visible platform positioned at the opposite end.

#### 2.2.3. Nociceptive behaviours elicited by intraplantar injection of algogens

Following habituation to individual perspex observation boxes (25 × 20 × 20 cm) for between 1 and 3 h, mice received an intraplantar injection (20 μl) of either the $B_1$ receptor agonist des[Arg$^{10}$]kallidin (300 nmol), the $B_2$ receptor agonist bradykinin (10 nmol), formalin (2.5%) or vehicle (saline or phosphate buffered saline) into one hind paw. The number of flinches and the duration of leg raising and licking of the injected paw were recorded continuously immediately thereafter for 15 min (bradykinin receptor agonists) or 35 min (early and late phase response to formalin).

Between six and nine control or transgenic mice received each algogen.

In a separate experiment, the effect of intraplantar coinjecti n of des-Arg$^9$[Leu$^8$]bradykinin (0.3 nmol) with formalin (2.5%) on the early and late phase nociceptive response was examined in *Bk2r*$^{-/-}$ mice.

#### 2.2.4. Induction of thermal hyperalgesia by intraplantar injection of carrageenan or complete Freund's adjuvant

Thermal hyperalgesia in mice was measured using a modified paw flick test using the apparatus described by Rupniak et al. (1995). Mice were habituated on glass tables (1 × 2 m) under opaque perspex boxes (10 × 10 × 10 cm) for at least 3 h in order to reduce exploratory activity. A mobile radiant heat source, located 2.5 cm beneath the glass surface, was pre-calibrated to give a baseline paw withdrawal latency of approximately 12 s. Response latencies were determined for one hind foot on three occasions and the mean was recorded as the baseline for each animal. Mice were then allocated to drug treatments ensuring that baseline latencies were balanced across groups. For carrageenan experiments, the mice were then returned to their home cages overnight. On the following day, they received an intraplantar injection (20 μl) of either carrageenan (0.6 mg) or saline into one hind paw, and were immediately placed on the glass tables to habituate as described above. Paw flick latency was reassessed for the inflamed paw 3 h later. At the end of this experiment, the animals were killed and both hind feet were amputated at the ankle and placed in pre-weighed vials for measurement of paw oedema.

For experiments examining the more prolonged inflammation induced by complete Freund's adjuvant (0.1%), mice received an intraplantar injection (30 μl) of the adjuvant or saline into one hind paw immediately after determination of the baseline paw flick latency. Animals were then returned to their home cages and the latency to withdraw the inflamed paw from the thermal stimulus was reassessed 4 days later as described above. Data are presented as the mean difference in paw flick latency for each animal before and after the induction of inflammation.

### 2.3. Preparation of test compounds

Indomethacin (Merck Research Laboratories, Rahway, USA) was dissolved in 1% sodium bicarbonate or suspended in 0.5% methocel prior to oral or intraperitoneal administration, respectively. Peptide bradykinin receptor agonists and antagonists (bradykinin, des-[Arg$^9$]bradykinin, des[Arg$^{10}$]kallidin and des-Arg$^9$[Leu$^8$]bradykinin; Bachem) were dissolved in distilled water and frozen at −20°C in aliquots at the required concentration until use.

### 2.4. Statistical analysis

Where necessary, data were subjected to square root or logarithmic transformation in order to achieve normality and homogeneity of variance, prior to one-way analysis of variance. If appropriate, data from drug-treated animals were compared with control animals using Dunnett's or Newman-Keuls multiple comparison *t*-tests.

## 3. Results

### 3.1. Antinociceptive activity of des-Arg$^9$[Leu$^8$]bradykinin in mice and rats

#### 3.1.1. Inhibition of late phase nociceptive response to formalin in normal mice

Intraplantar injection of formalin (2.5%) in BKTO mice elicited a biphasic nociceptive response comprising flinching, leg raising and licking of the injected paw. During the late phase response, 20–30 min after injection of formalin, the duration of paw licking was attenuated to a similar extent (approximately 50%) in animals pretreated with indomethacin (30 mg/kg i.p.) or 30 nmol/kg s.c. of des-Arg$^9$[Leu$^8$]bradykinin compared with saline-treated control mice ($F_{5,63} = 5.47$, $P < 0.001$). The active dose window for des-Arg$^9$[Leu$^8$]bradykinin was extremely narrow since neither lower (3 or 10 nmol/kg) nor higher (100 nmol/kg) doses significantly inhibited formalin-induced licking (Fig. 1).

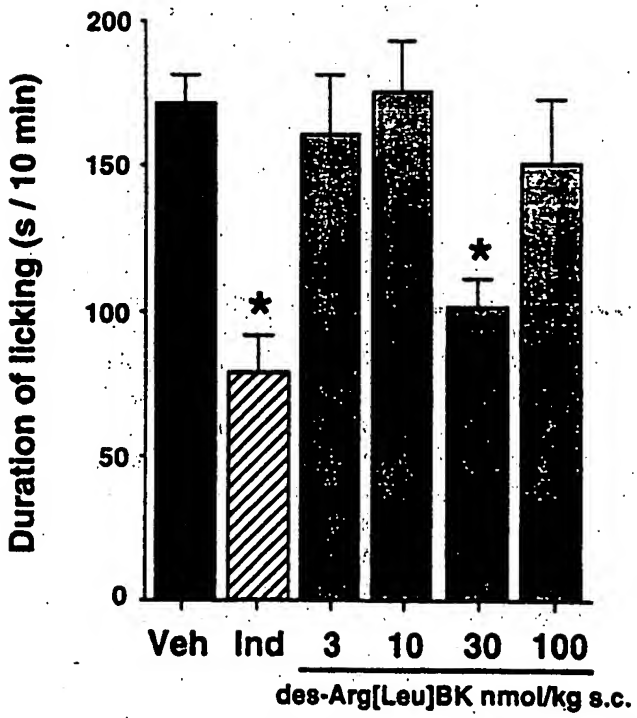


Fig. 1. Effect of indomethacin (30 mg/kg i.p.) or des-Arg$^9$[Leu$^8$]bradykinin (3–100 nmol/kg s.c.) on the late phase paw licking response induced by formalin in normal BKTO mice. Animals received an intraplantar injection f formalin (20 μl of 2.5% solution) 10 min after the test compounds. The duration of licking was recorded throughout the period 10–20 min thereafter. Data were subjected to one way ANOVA f llowed by Dunnett's *t*-test ($n = 12$ per group). *$P \leq 0.05$ compared with vehicle treatment.

*3.1.2. Inhibition of carrageenan-induced hyperalgesia in rats*

Intraplantar injection of carrageenan (4.5 mg 3 h previously) induced marked paw oedema and hyperalgesia to compression of the inflamed paw. Like indomethacin (10 mg/kg p.o.), treatment with des-Arg$^9$[Leu$^8$]bradykinin (1–30 nmol/kg i.v.) caused a dose-dependent and complete reversal of carrageenan-induced hyperalgesia ($F_{6,64} = 9.04$, $P < 0.001$). However, administration of a higher dose of des-Arg$^9$[Leu$^8$]bradykinin (100 nmol/kg) caused only a 40% inhibition of hyperalgesia which failed to reach significance (Fig. 2).

*3.1.3. Ankle flexion test in monoarthritic rats*

Intra-articular injection of Freund's adjuvant 7–8 days previously caused erythema and swelling of the ankle joint. Gentle flexion and extension of the inflamed (but not the non-inflamed contralateral) ankle elicited vocalisations which were recorded as a measure of hyperalgesia. In rats with pronounced hyperalgesia (vocalisation response elicited on more than five out of 10 combined flexions and extensions), administration of indomethacin (10 mg/kg p.o., 1 h previously) partially attenuated hyperalgesia by 30% compared with vehicle-treated animals (number of vocalisations: saline = 9.1 ± 0.4, compared with 6.3 ± 0.7; $F_{4,75} = 7.14$, $P < 0.001$). The number of vocalisations was not significantly attenuated by similar pretreatment intravenously with des-Arg$^9$[Leu$^8$]bradykinin (number of vocalisations: 1 nmol/kg = 9.1 ± 0.4; 10 nmol/kg = 8.5 ± 0.5; 30 nmol/kg = 9.9 ± 0.1).

In rats with less marked hyperalgesia (vocalisation scores <5), intraven us injecti n of the $B_1$ receptor agonist des-[Arg$^9$]bradykinin (100 nm l/kg, 1 h previously) doubled the number of vocalisations elicited by flexion and extension of the inflamed paw (number of vocalisations: saline = 3.6 ± 0.5, compared with 7.0 ± 0.7 for des-[Arg$^9$]bradykinin treated rats; $F_{1,18} = 13.62$, $P < 0.002$). In contrast, vocalisations were not induced by passive flexion and extension of the non-inflamed, contralateral joint in the same animals (number of vocalisations: saline = 0 ± 0, compared with 0.2 ± 0.1 for des-[Arg$^9$]bradykinin treated rats; $F_{1,17} = 2.55$, $P = 0.1$).

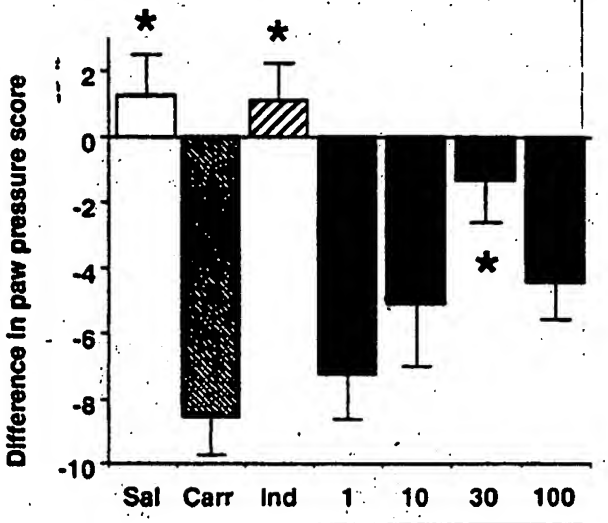


Fig. 2. Effect of indomethacin (10 mg/kg p.o.) or des-Arg$^9$[Leu$^8$]bradykinin (1–100 nmol/kg i.v.) on carrageenan-induced mechanical hyperalgesia in rats. Test compounds were administered 2 h after intraplantar injection of carrageenan (4.5 mg) and the change in paw pressure thresholds was determined 1 h later. Data were subjected to one way ANOVA followed by Dunnett's *t*-test ($n = 10$ per group). *$P \leq 0.05$ compared with vehicle treatment.

*3.2. Sensorimotor function and nociception in Bk2r$^{-/-}$ mice*

*3.2.1. Spinal reflexes and tests of sensorimotor co-ordination*

Bk2r$^{-/-}$ mice (J129 × C57 hybrids) were indistinguishable in their appearance, spontaneous behaviour and body weights from age and sex-matched wild-type or heterozygous *Bk2r*$^{+/-}$ control animals. All *Bk2r*$^{-/-}$ and control mice exhibited normal placing and righting reflexes and hindlimb reflex extension, and had no difficulty in maintaining their balance on beams for the full 120-s trial duration. Similarly, *Bk2r*$^{-/-}$ mice were able to maintain their balance on a revolving rota-rod treadmill for the same duration as control mice (time on rota-rod (s): controls = 47.7 ± 11.8, compared with 33.4 ± 10.5 for *Bk2r*$^{-/-}$ mice; $F_{1,18} = 0.82$, $P = 0.38$), and had normal swimming speeds for escape onto a platform in the water tank (escape latency (s): controls = 8.9 ± 2.1, compared with 9.6 ± 2.1 for *Bk2r*$^{-/-}$ mice; $F_{1,18} = 0.06$, $P = 0.81$).

*3.2.2. Nociceptive response to intraplantar injection of algogens and inflammatory agents in Bk2r$^{-/-}$ mice*

*3.2.2.1. Bradykinin receptor agonists.* In wild-type and heterozygous *Bk2r*$^{+/-}$ control J129 × C57 mice, intraplantar injection of the $B_2$ receptor agonist bradykinin (10 nmol) elicited a vigorous, short-lasting (<10 min total duration) aversive behavioural response consistent with the perception of an algogenic stimulus. The most prominent features were repetitive flinching and leg raising so that the affected limb was no longer weight-bearing. In contrast, injection of the same dose of bradykinin in *Bk2r*$^{-/-}$ mice failed to elicit flinching, leg raising or any other observable behavioural response. The difference in the response to bradykinin in control and *Bk2r*$^{-/-}$ mice was highly significant (flinching: $F_{1,13} = 12.61$, $P = 0.004$; leg raising: $F_{1,13} = 12.60$, $P = 0.004$; Fig. 3).

Similarly, intraplantar injection of a high dose of the $B_1$ receptor preferring agonist des[Arg$^{10}$]kallidin (300 nmol) elicited flinching in control animals, but not in *Bk2r*$^{-/-}$ mice ($F_{1,10} = 11.95$, $P = 0.006$). Leg raising was also induced by des[Arg$^{10}$]kallidin in normal mice, but this response was less marked than that observed after injection of bradykinin. In *Bk2r*$^{-/-}$ mice, leg raising elicited

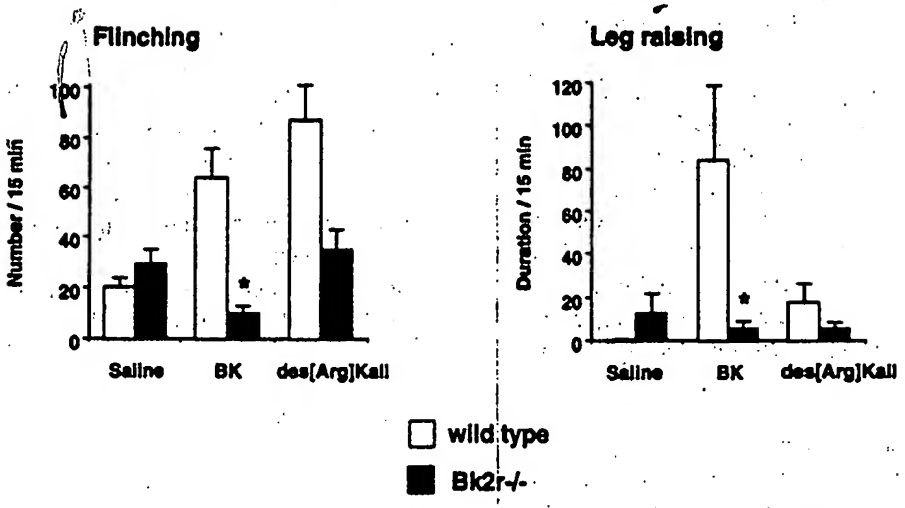


Fig. 3. Aversive behaviours elicited by intraplantar injection of bradykinin (10 nmol) or des[Arg$^{10}$]kallidin (300 nmol) in wild type and *Bk2r*$^{-/-}$ mice. Behaviours were recorded by direct observation for 15 min. Data were subjected to one-way ANOVA followed by Dunnett's *t*-test ($n$ = 6–9 per group). *$P \leq 0.05$ compared to wild type control animals.

by des[Arg$^{10}$]kallidin appeared to be of shorter duration, but the difference between control and transgenic animals failed to reach statistical significance ($F_{1,10} = 3.42$, $P = 0.09$; Fig. 3).

*3.2.2.2. Early and late phase response to formalin.* In J129 control mice, intraplantar injection of formalin (2.5%) elicited a biphasic nociceptive response of flinching, leg raising and paw licking. The early phase peaked during the first 10 min after injection of formalin, and the late phase lasted until 35 min after formalin. The response to formalin was considerably less vigorous in this strain than in the albino BKTO mice described above, and it was therefore necessary to sum the duration of leg raising and paw licking in order to permit reliable quantitative assessment. The early and late phase response to formalin was indistinguishable in J129 wild-type and *Bk2r*$^{-/-}$ mice of the same strain (early phase: $F_{1,13} = 0.44$, $P = 0.51$; late phase: $F_{1,13} = 0.18$, $P = 0.68$; Fig. 4).

In experiments examining the ability of the $B_1$ receptor antagonist des-Arg$^9$[Leu$^8$]bradykinin to inhibit nociception in *Bk2r*$^{-/-}$ mice, it was found that handling animals from the J129 strain for systemic drug administration prior to challenge with formalin caused a profound disruption of the behavioural response to the algogen. For this reason it was necessary to administer des-Arg$^9$[Leu$^8$]bradykinin by intraplantar coinjection (0.3 nmol) with the formalin. The $B_1$ receptor antagonist markedly attenuated the late, but not the early, phase response to formalin in *Bk2r*$^{-/-}$ mice (early phase: $F_{1,16} = 2.01$, $P = 0.17$; late phase: $F_{1,16} = 4.89$, $P = 0.04$; Fig. 5).

*3.2.2.3. Thermal hyperalgesia induced by carrageenan or Freund's adjuvant.* The paw withdrawal latency following exposure to a noxious thermal stimulus was not different in control and *Bk2r*$^{-/-}$ mice that had not received injections of inflammatory agents (paw flick latency (s): control, 14.3 ± 0.8, compared with 14.7 ± 0.8 in *Bk2r*$^{-/-}$ mice, $F_{1,20} = 0.07$, $P = 0.80$).

There were marked differences in the induction of acute thermal hyperalgesia and paw oedema by carrageenan in control and *Bk2r*$^{-/-}$ mice. Intraplantar injection of carrageenan (0.6 mg), but not an equivalent volume of saline (20 μl), 3 h previously reduced the paw withdrawal latency compared with pre-injection baseline values in control J129 mice, but not in J129 *Bk2r*$^{-/-}$ mice ($F_{3,28} = 4.69$, $P = 0.009$; Table 1). Paw oedema was also considerably less pronounced in *Bk2r*$^{-/-}$ mice than in control animals injected with carrageenan ($F_{3,28} = 57.64$, $P < 0.001$; Table 1).

In contrast to the results observed using carrageenan, the induction of chronic thermal hyperalgesia by intraplantar injection of complete Freund's adjuvant (0.1%) 3 days pre-

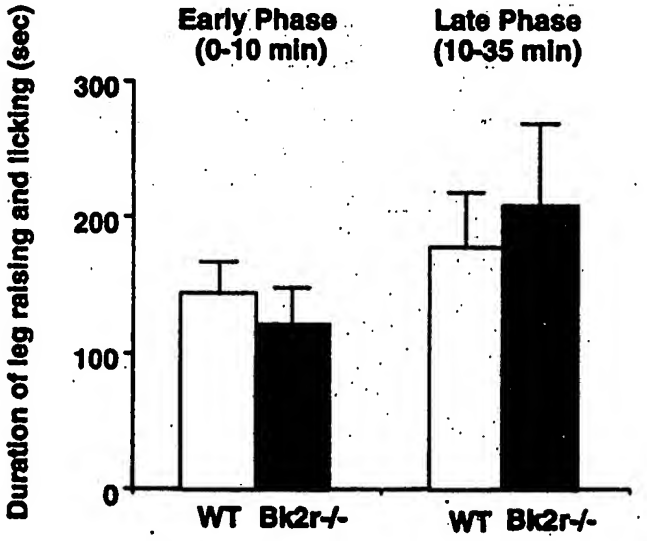


Fig. 4. Early and late phase nociceptive response to intraplantar injection of formalin (2.5% in 20 μl) in wild type and *Bk2r*$^{-/-}$ mice. Behaviours were recorded by direct observation for 35 min. Data were subjected to one way ANOVA followed by Dunnett's *t*-test ($n$ = 6–9 per group).

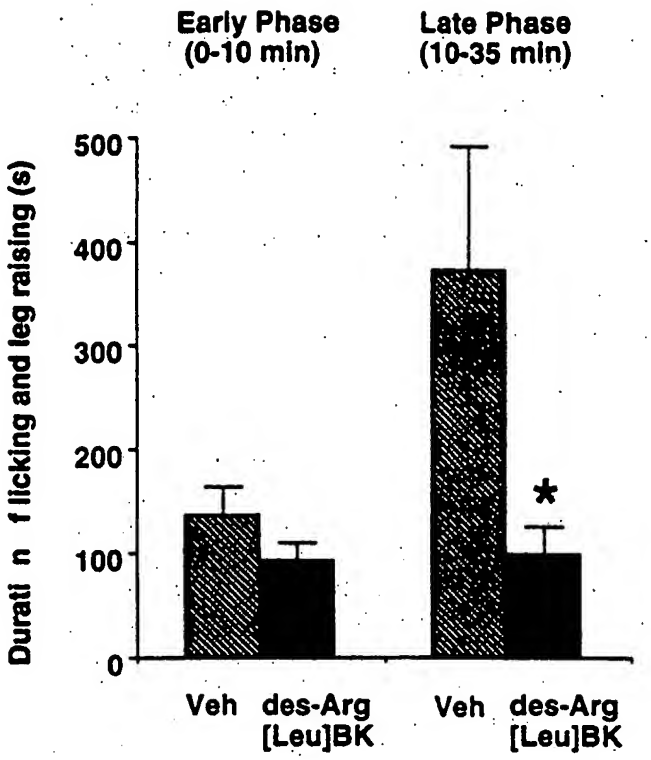


Fig. 5. Inhibition of late phase response to intraplantar injection of formalin (2.5%) by des-Arg$^9$[Leu$^8$]bradykinin (0.3 nmol) in *Bk2r*$^{-/-}$ mice. Data were subjected to one way ANOVA followed by Dunnett's *t*-test ($n$ = 6–9 per group). *$P \leq 0.05$ compared with vehicle treatment.

viously was indistinguishable in wild type control and *Bk2r*$^{-/-}$ mice (Table 2). Paw oedema was not quantified in this experiment but was evident in both groups of mice on visual inspection.

## 4. Discussion

The present studies were aimed at clarifying the relative contributions of bradykinin $B_1$ and $B_2$ receptors in acute and chronic inflammatory hyperalgesia. The experiments examined nociceptive responses in animals treated with the peptide $B_1$ receptor antagonist des-Arg$^9$[Leu$^8$]bradykinin and in transgenic mice in which the $B_2$ receptor had been disrupted.

Table 1

Induction of thermal hyperalgesia and paw oedema by intraplantar injection of carrageenan (0.6 mg) or saline (20 μl) 3 h previously in normal and *Bk2r*$^{-/-}$ J129 mice

| Group and treatment | Paw flick latency (difference from pre-injection baseline; s) | Paw oedema (difference from non-injected paw; mg) |
|---|---|---|
| Control + saline | −1.1 ± 1.2 | 2.66 ± 1.5 |
| Control + carrageenan | −5.7 ± 0.7* | 51.6 ± 3.7* |
| *Bk2r*$^{-/-}$ + saline | −1.6 ± 0.7 | 6.2 ± 2.4 |
| *Bk2r*$^{-/-}$ + carrageenan | −2.5 ± 0.6** | 26.2 ± 3.7*,** |

*$P \leq 0.05$ compared to saline treatment; **$P \leq 0.05$ compared to wild type control animals.
Values are the mean ± 1 SEM for 7–8 animals. Data were subjected to one-way ANOVA, foll wed by Dunnett's multiple comparison *t*-tests.

Table 2

Induction of thermal hyperalgesia by intraplantar injection of complete Freund's adjuvant (CFA; 0.1%) or saline (30 μl) 3 days previously in normal and *Bk2r*$^{-/-}$ J129 mice

| Group and treatment | Paw flick latency (difference from pre-injection baseline; s) |
|---|---|
| Control + saline | 4.5 ± 1.2 |
| Control + CFA | −7.9 ± 0.8* |
| *Bk2r*$^{-/-}$ + saline | 1.8 ± 2.3 |
| *Bk2r*$^{-/-}$ + CFA | −7.4 ± 1.6* |

*$P \leq 0.05$ compared to saline treatment.
Values are the mean ± 1 SEM for 5–6 animals. Data were subjected to one-way ANOVA, followed by Dunnett's multiple comparison *t*-tests.

The $B_1$ receptor antagonist des-Arg$^9$[Leu$^8$]bradykinin exhibited antinociceptive activity in conscious animal assays of acute inflammatory hyperalgesia that are sensitive to NSAIDs. Like indomethacin, systemic administration of des-Arg$^9$[Leu$^8$]bradykinin fully reversed carrageenan-induced mechanical hyperalgesia in rats and attenuated aversive behaviours elicited by intraplantar injection of formalin in normal mice. The antinociceptive effects of des-Arg$^9$[Leu$^8$]bradykinin were not clearly dose-related, reaching significance at only one dose (30 nmol/kg). This narrow active dose window for des-Arg$^9$[Leu$^8$]bradykinin resembles that observed previously in the formalin assay (Correa and Calixto, 1993), and may be attributable to the partial agonist activity reported recently for this $B_1$ receptor antagonist (Allogho et al., 1995). Partial agonism, combined with metabolic lability, are major limitations of des-Arg$^9$[Leu$^8$]-bradykinin, and also of the currently available peptide $B_2$ receptor antagonists (Steranka et al., 1987; Steranka et al., 1989; Kindgen-Milles and Klement, 1992; Rhaleb et al., 1991). Stable non-peptide bradykinin $B_1$ receptor antagonists are therefore required in order to fully define their clinical potential and the physiological roles of these receptors in vivo.

In an assay examining chronic inflammation, in which hyperalgesia was assessed by recording vocalisations elicited by flexion and extension of the inflamed limb of monoarthritic rats, indomethacin caused a partial reduction of the number of vocalisations, but an anti-algesic effect of des-Arg$^9$[Leu$^8$]bradykinin was not detected. However, intravenous injection of the $B_1$ receptor agonist des-[Arg$^9$]bradykinin markedly increased the number of vocalisations elicited by manipulation of the inflamed joint but not of the non-inflamed contralateral limb, suggesting the local induction of $B_1$ receptors. This observation is in agreement with the findings of Davis and Perkins (1993) that intra-articular injection of des-[Arg$^9$]bradykinin exacerbated adjuvant-induced arthritis as assessed by weight bearing in rats. The failure to detect antinociceptive activity of des-Arg$^9$[Leu$^8$]bradykinin using the vocalisation assay

employed here contrasts with the ability of this peptide to increase the load tolerated by monoarthritic animals in the studies of Davis and Perkins (1993) and Perkins et al. (1993). This discrepancy may reflect differences in the sensitivity of vocalisation and weight bearing as nociceptive endpoints, coupled with the difficulties of establishing the antinociceptive dose range of des-Arg$^9$[Leu$^8$]bradykinin described above.

In view of the known algogenic and pro-inflammatory effects of bradykinin that are believed to be mediated via $B_2$ receptor activation, disruption of this receptor produced surprisingly subtle changes in nociception in *Bk2r*$^{-/-}$ mice. As would be expected following deletion of this gene, *Bk2r*$^{-/-}$ mice failed to exhibit any nociceptive behavioural response to intraplantar injection of bradykinin, confirming that the direct activation of nociceptors by bradykinin agonists involves the $B_2$ rather than the $B_1$ receptor, at least in n n-inflamed tissues. Similarly, the nociceptive response elicited by the $B_1$ receptor-preferring agonist des-[Arg$^{10}$]-kallidin in wild type mice was absent in *Bk2r*$^{-/-}$ mice, indicating that the selectivity of this agonist is lost at high doses. The localisation of $B_2$ receptors on sensory neurones is consistent with the ability of HOE 140 and other peptide $B_2$ receptor antagonists to inhibit nociception elicited by bradykinin in animals and man (Steranka et al., 1987; Whalley et al., 1987; Heapy et al., 1993). In other studies it was found that bradykinin, but not the $B_1$ receptor agonists des-[Arg$^9$]-bradykinin or des-[Arg$^{10}$]kallidin, could activate primary afferents in a spinal nociceptive reflex preparation (Dray et al., 1992), even following tissue damage and inflammation (Davis et al., 1996). Moreover, unlike the activation of sensory neurones elicited by bradykinin, there was no response to $B_1$ receptor agonists in dorsal root ganglion neurones taken from normal animals or rats pretreated with inflammatory mediators (Davis et al., 1996). These findings suggest that the $B_1$ receptors that contribute to inflammatory hyperalgesia may not be located on sensory neurones, but may be expressed by other cells that release mediators that sensitise or directly activate nociceptors. However, other evidence indicates that $B_1$ receptors may be also expressed in neuronal tissues. Thus, $B_1$ receptor mediated responses were induced by cytokines in isolated superior cervical ganglia after inhibition of peptidase activity with captopril (Seabrook et al., 1995), although this was not observed in the absence of captopril (Babbedge et al., 1995). More recent studies also indicate the presence of $B_1$ receptor mRNA in superior cervical and dorsal root ganglia of normal and *Bk2r*$^{-/-}$ mice (Seabrook et al., unpublished observations).

The *Bk2r*$^{-/-}$ mice also failed to develop paw oedema or thermal hyperalgesia in response to intraplantar injection of carrageenan, indicating that the action of bradykinin at $B_2$ receptors is an essential initial step in the inflammatory response to this polysaccharide. However, the ability of des-Arg$^9$[Leu$^8$]bradykinin to inhibit carrageenan-induced hyperalgesia in normal animals (see above) indicates that both $B_1$ and $B_2$ receptors are involved in maintaining the ensuing inflammation and hyperalgesia. Therefore, it appears that blockade of both $B_1$ and $B_2$ receptors may contribute to the inhibition of carrageenan-induced oedema and thermal hyperalgesia by peptide antagonists such as NPC 567 (Costello and Hargreaves, 1989).

In contrast to their abnormal response to bradykinin agonists and to carrageenan, *Bk2r*$^{-/-}$ mice exhibited unimpaired sensorimotor and neurological function, and were visibly indistinguishable from heterozygous or wild type control animals. Spinal nociceptive reflexes, assessed using a paw flick test, appeared normal in *Bk2r*$^{-/-}$ mice, suggesting that bradykinin may not be involved in the activation of thermal nociceptors in the absence of inflammation or tissue damage. Moreover, activation of $B_2$ receptors by bradykinin is not required for all acute and chronic nociceptive and inflammatory reactions, since nociception and inflammatory hyperalgesia elicited by intraplantar injection of formalin or Freund's adjuvant were indistinguishable in *Bk2r*$^{-/-}$ and wild type mice. This finding was unexpected in view of reports that peptide $B_2$ receptor antagonists, such as HOE 140 and NPC 567, could attenuate nociceptive responses evoked by formalin (Correa and Calixto, 1993) and increase the load tolerated by the arthritic joints of experimental rats (Davis and Perkins, 1994a), although these effects were typically modest and seen within only a narrow dose window. The apparently normal response to formalin and Freund's adjuvant in *Bk2r*$^{-/-}$ mice suggests that other pro-inflammatory mediator(s) can by-pass $B_2$ receptor activation; if this is also true of human pathological conditions, then the clinical efficacy of $B_2$ receptor antagonists as anti-inflammatory and analgesic drugs may be limited. Moreover, in several animal assays of persistent inflammation that may be argued to be of most relevance for clinical conditions, the effects of HOE 140 were weak or absent compared with those of des-Arg$^9$[Leu$^8$]bradykinin (Perkins and Kelly, 1993; Perkins et al., 1993; Davis and Perkins, 1994a Davis and Perkins, 1994b). Interestingly, the antinociceptive effect of des-Arg$^9$[Leu$^8$]bradykinin against the late phase response to formalin was still demonstrable in *Bk2r*$^{-/-}$ mice in the present studies, indicating that induction of $B_1$ receptors is also not dependent on $B_2$ receptor stimulation, and can occur rapidly at the site of inflammation (<30 min). This finding has recently been replicated in *Bk2r*$^{-/-}$ mice of the C57 strain that exhibit a more pronounced nociceptive response to formalin (unpublished observations). Thus, $B_1$ receptors appear to contribute significantly to nociception associated with both acute and chronic inflammatory insults.

In summary, the present studies confirm and extend previous findings that the $B_1$ receptor antagonist des-Arg$^9$[Leu$^8$]bradykinin exhibits antinociceptive activity in assays of acute and chronic inflammatory hyperalgesia. Metabolically stable, non-peptide antagonists that are devoid of partial agonist activity are required to further examine the possible clinical potential of blocking the $B_1$ receptor. Phenotypic characterisation of *Bk2r*$^{-/-}$ mice indicates that $B_2$ receptor

stimulation is required for some, but not all, inflammatory responses and that the clinical potential of $B_2$ receptor antagonists to treat chronic pain and inflammation may therefore be limited.

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